BIOLOGY OF REPRODUCTION 66, 1193–1202 (2002)

Exhibit A

Analysis of the Roles of RGD-Binding Integrins, α_4/α_9 Integrins, α_6 Integrins, and CD9 in the Interaction of the Fertilin β (ADAM2) Disintegrin Domain with the Mouse Egg Membrane[1]

Xiaoling Zhu and Janice P. Evans[2]

Department of Biochemistry and Molecular Biology, Division of Reproductive Biology, Johns Hopkins University, Bloomberg School of Public Health, Baltimore, Maryland 21205

ABSTRACT

Fertilin β (also known as ADAM2), a mammalian sperm protein that mediates gamete cell adhesion during fertilization, is a member of the ADAM protein family whose members have disintegrin domains with homology to integrin ligands found in snake venoms. Fertilin β utilizes an ECD sequence within its disintegrin domain to interact with the egg plasma membrane; the Asp is especially critical. Based on what is known about different integrin subfamilies and their ligands, we sought to characterize fertilin β binding sites on mouse eggs, focusing on integrin subfamilies that recognize short peptide sequences that include an Asp residue: the $\alpha_5/\alpha_8/\alpha_v/\alpha_{IIb}$ or RGD-binding subfamily ($\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, and $\alpha_{IIb}\beta_3$) and the α_4/α_9 subfamily ($\alpha_4\beta_1$, $\alpha_9\beta_1$, and $\alpha_4\beta_7$). We tested peptide sequences known to perturb interactions mediated by these integrins in two different assays for fertilin β binding. Peptides with the sequence MLDG, which perturb α_4/α_9 integrin-mediated interactions, significantly inhibit fertilin β binding to eggs, which suggests a role for a member of this integrin subfamily as a fertilin β receptor. RGD peptides, which perturb $\alpha_5/\alpha_8/\alpha_v/\alpha_{IIb}$ integrin-mediated interactions, have partial inhibitory activity. The anti-α_6 antibody GoH3 has little or no inhibitory activity. An antibody to the integrin-associated tetraspanin protein CD9 inhibits the binding of a multivalent presentation of fertilin β (immobilized on beads) but not soluble fertilin β, which we speculate has implications for the role of CD9 in the strengthening of fertilin β-mediated cell adhesion but not in initial ligand binding.

fertilization, gamete biology, ovum, sperm

INTRODUCTION

The interaction of gamete plasma membranes is mediated by cell adhesion molecules on the sperm and egg. Current evidence suggests that three ADAM (a disintegrin and metalloprotease domain) proteins, fertilin α (ADAM1), fertilin β (ADAM2), and cyritestin (ADAM3), participate in mammalian sperm-egg adhesion [1–8]. ADAMs, most notably the disintegrin domains in these proteins, share sequence homology with snake venom disintegrins and metalloproteases [9–11]. Several of these snake venom polypeptides interact with certain integrins via sequences in a region of the disintegrin domain, called the "disintegrin loop," because an adhesion-mediating tripeptide sequence, RGD, is presented at the end of an extended loop [12, 13]. Very few ADAMs have an RGD sequence in the disintegrin loop. Studies of point-mutated versions of the fertilin β disintegrin loop have demonstrated that the sequence ECD, especially the Asp residue, is the critical region of mouse fertilin β for mediating gamete cell adhesion during fertilization [14, 15].

One or more integrins on the egg surface have been proposed to serve as a receptor for fertilin β as well as other sperm ADAMs. Integrins are heterodimeric membrane receptors that mediate cell-matrix and cell-cell interactions. To date, 18 different α subunits and 8 β subunits have been identified in vertebrates. These combine to make 24 different α/β combinations, all with different ligand specificities. Integrins can be grouped into five distinct subfamilies based on sequence homologies between integrin α subunits and on ligand specificities (Fig. 1). Although $\alpha_6\beta_1$ was first proposed to be the egg receptor for fertilin β [16, 17], other data indicate that α_6 on the egg plasma membrane is not essential for fertilization or for fertilin β binding to eggs [2, 18].

Based on what is known about integrin subfamilies and the ligands that they recognize, we undertook a study to characterize the fertilin β binding sites on mouse eggs, focussing on candidate integrins that interact with ligands with similarities to fertilin β. As noted above, an ECD sequence in the fertilin β disintegrin domain mediates the interaction of fertilin β with the egg plasma membrane; the Asp residue within this motif appears to be critical [14, 15]. Two integrin subfamilies recognize short peptide sequences that include an Asp residue (Fig. 1). One of these is the $\alpha_5/\alpha_8/\alpha_v/\alpha_{IIb}$ integrin subfamily ($\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, and $\alpha_{IIb}\beta_3$), also known as RGD-binding integrins (Fig. 1). As this name implies, these integrins recognize RGD tripeptide sequences in a variety of ligands, including fibronectin, vitronectin, fibrinogen, and other molecules. The interactions between RGD-binding integrins and their ligands can be disrupted with RGD-containing synthetic peptides and snake venom disintegrins [9, 10, 19]. In addition, three of the five integrins implicated as receptors for ADAMs are members of the RGD-binding subfamily: $\alpha_v\beta_3$ [20–22], $\alpha_v\beta_5$, [23], and $\alpha_5\beta_1$ [21]. These integrins interact with ADAM15, ADAM9, and ADAM23. ADAM9 [23] and ADAM23 [22] do not have RGD sequences in their disintegrin domains, but instead have ECD sequences, such as fertilin β. The human homologue of ADAM15 has the sequence RGDCD, with an RGD tripeptide as well as the sequence DCD, which is similar to and aligns with the ECD, which is found in fertilin β and other ADAMs.

[1]This research was supported by grant HD 37696 from the National Institutes of Health (NIH) and by a Faculty Innovation Award from the Johns Hopkins Bloomberg School of Public Health. X.Z. was supported by NIH training grant HD 07276.

[2]Correspondence: Janice P. Evans, Division of Reproductive Biology, Department of Biochemistry and Molecular Biology, Johns Hopkins University, Bloomberg School of Public Health, 615 N. Wolfe St., Room 3606, Baltimore, MD 21205. FAX: 410 614 2356; e-mail: jpevans@jhsph.edu

Received: 4 October 2001.
First decision: 5 November 2001.
Accepted: 19 November 2001.

ISSN: 0006-3363. http://www.biolreprod.org

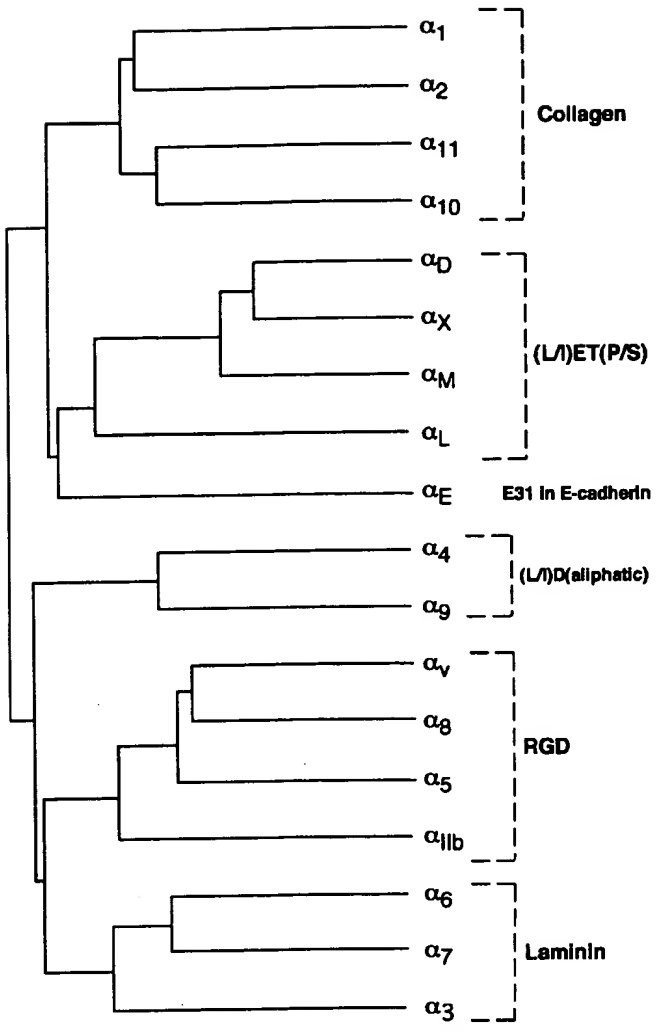

FIG. 1. Sequence similarities and ligand specificities of integrin α subunits. The similarity tree is based on previously published similarity trees [64, 65]. The general ligand specificities of each of the subfamilies are noted in the figure (more details are discussed in [26]; note that the consensus sequence given for α_4 and α_9 applies primarily to α_4 integrins).

Another integrin subfamily, the α_4/α_9 integrins ($\alpha_4\beta_1$, $\alpha_9\beta_1$, and $\alpha_4\beta_7$), recognizes short peptide sequences that include an Asp residue (Fig. 1). The adhesion-mediating sites in a subset of α_4/α_9 ligands include an Asp residue (fibronectin, LDVP; VCAM-1, IDSP; MadCAM-1, LDTS; tenascin-C, AEIDGIEL; see [24–26] and references therein). In the ligands for which the structure is known, the Asp residues (Asp40 in VCAM-1 [27] and Asp42 in MadCAM-1 [28]) are presented on protruding loops, similar to the presentation of the RGD tripeptide in fibronectin [29] and disintegrins [12, 13]. In addition, $\alpha_9\beta_1$ interacts with sequences that are similar to the ECD in mouse fertilin β: RGDCD in human ADAM15, TDDCD in mouse ADAM15, SNSCD in human and mouse ADAM12, and SGACD in a mutated form of human ADAM15 [30]. The interactions of $\alpha_4\beta_1$ and $\alpha_9\beta_1$ with one ligand, VCAM-1, can be disrupted with the dimeric snake disintegrins EC3 and EC6 and with peptides containing the sequence MLDG, corresponding to the disintegrin loop sequences from the EC3B and EC6A subunits [31–33]. The MLD in these proteins aligns with the RGD in other snake disintegrins. Because $\alpha_4\beta_1$ recognizes an Asp-containing sequence (IDS in VCAM-1) and this interaction is disrupted by an MLDG-containing peptide, we hypothesized that an MLDG-containing peptide might perturb the ECD-mediated interaction of fertilin β with binding sites on the egg membrane.

In this study we examine whether the binding of recombinant fertilin β disintegrin domain to mouse eggs can be perturbed by RGD-containing and MLDG-containing peptides. These sequences are presented on bacterial alkaline phosphatase (BAP), because we have observed that ECD peptide sequences presented at the termini of fusion proteins are more effective inhibitory reagents than are synthetic peptides [15]. Fertilin β binding to zona pellucida (ZP)-free eggs is assessed using two different assays and presentations of fertilin β: "soluble fertilin β" (diluted in culture medium; egg-associated protein is detected with a quantitative luminometric immunoassay [2]) and "bead-immobilized fertilin β" (fluorescent beads coated with recombinant fertilin β medium; egg-associated beads are detected by fluorescence microscopy [34]). Results with the RGD and MLDG peptides in these two assays prompted us to reexamine two other egg molecules that have been implicated in fertilization and the interactions of sperm and fertilin β with the egg: the integrin $\alpha_6\beta_1$ [14, 16, 17], and the tetraspanin CD9 [35–38]. Our data implicate a member of the α_4/α_9 subfamily of integrins in the interaction of fertilin β with the egg membrane. We also observe that bead-immobilized fertilin β appears to interact with a binding site that is sensitive to perturbation by an anti-CD9 antibody, whereas soluble fertilin β interacts with a site that is not perturbed by this antibody, which is similar to what we have observed for fertilin α [39]. These data raise the possibility that the binding of fertilin β in a multimeric context (i.e., multiple molecules coating a bead) to eggs requires CD9 function, possibly via a receptor that is a component of a multiprotein complex containing CD9 (such tetraspanin-containing complexes are called "tetraspanin webs" [40]). In contrast, binding of fertilin β in a monomeric context (i.e., soluble in culture medium) to eggs does not require CD9 function. These data will be discussed with respect to ligand-receptor interactions as compared to adhesion strengthening.

MATERIALS AND METHODS

Production of Recombinant Forms of the Fertilin β Disintegrin Domain

Mouse fertilin β disintegrin domain (βD) and disintegrin loop (βDL) were generated as fusion proteins with maltose binding protein (MBP) as previously described [15]. Mouse fertilin βD was also generated as a fusion protein with glutathione S-transferase (GST). To generate GST-fertilin βD, a DNA fragment encoding the mouse fertilin β disintegrin domain (nucleotides 1162–1431 of GenBank accession number U16242) was prepared by polymerase chain reaction (PCR) amplification from mouse fertilin β plasmid DNA [41] using *Pfu* polymerase (Promega, Madison, WI). The 5′ primer (5′-GCGGATCCCATCACCATCACCATCACAAGATGGCGGTCTGTG-3′) corresponded to nucleotides 1162–1177 of mouse fertilin β also contained a six-histidine (6-His) tag and a *Bam*HI restriction site. The 3′ primer (5′-GAGAGTCGACTTAACCGTTTTGAACAAAGAA-3′) corresponded to nucleotides 1414–1431 of mouse fertilin β and also included a stop codon and a *Sal*I site. The PCR product was digested with *Bam*HI and *Sal*I, and was cloned into pGEX-4T-1 vector (Amersham-Pharmacia Biotech Inc., Piscataway, NJ) according to standard protocols. The resulting plasmid (pGEX-4T-1-βD) was sequenced to verify the correct DNA sequence and in-frame cloning. To produce GST-fertilin βD, a 1-L culture of pGEX 4T-1-βD-carrying DH5α *Escherichia coli* was induced with 0.1 mM isopropylthiogalactoside (Sigma, St. Louis, MO) at 30°C for 4 h. The cells were pelleted and resuspended in 20 ml of cold PBS. The resuspension was sonicated on ice three times, 1 min each time.

TABLE 1. Oligonucleotide sequences used to generate BAP-presented peptides.

BAP-presented peptide	Oligonucleotide and deduced amino acid sequences
BAP-ECD	CTAGT GCC CAA GAT GAG TGT GAT GTC ACAG
	A CGG GTT CTA CTC ACA CTA GCAG TGTCCTAG
	A Q D E C D V T
BAP-ECE	CTAGT GCC CAA GAT GAG TGT GAG GTC ACAG
	A CGG GTT CTA CTC ACA CTC CAG TGTCCTAG
	A Q D E C E V T
BAP-ECA	CTAGT GCC CAA GAT GAG TGT GCA GTC ACAG
	A CGG GTT CTA CTC ACA CGT CAG TGTCCTAG
	A Q D E C A V T
BAP-RGD	CTAGT GGA CGT GGC GAT ACA CGT G
	A CCT GCA CCG CTA TGT GGA CCTAG
	G R G D T P
BAP-RGE	CTAGT GGT CGA GGA GAG ACT CCT G
	A CCA GCT CCT CTC TGA GGA CCTAG
	G R G E T P
BAP-RGA	CTAGT GGA CGT GGC GCC ACG CCT G
	A CCT GCA CCG CGG TGC GGA CCTAG
	G R G A T P
BAP-sRGD	CTAGT ACG GAT GGA CCT GGT CGC G
	A TGC CTA CCT GGA CCA GCG CCTAG
	T D G P G R
BAP-MLDG	CTAGC AAG CGA GCT ATG CTA GAC GGT CTC AAT GAT TAC G
	G TTC GCT CGA TAC GAT CTG CCA GAG TTA CTA ATG CCTAG
	K R A M L D G L N D Y
BAP-MAAG	CTAGC AAG CGA GCT ATG GCA GCC GGT CTC AAT GAT TAC G
	G TTC GCT CGA TAC CGT CGG CCA GAG TTA CTA ATG CCTAG
	K R A M A A G L N D Y

After centrifugation at 5000 × *g* for 20 min, the supernatant was applied to a 1-ml glutathione column. The column was washed with ~50 bed volumes of PBS, then GST-fertilin βD was eluted from the column with 10 mM of reduced glutathione in 10 mM Tris-HCl, pH 7.5. The GST-fertilin βD protein ran at an M_r = 39 000 under reducing conditions and M_r = 36 500 under nonreducing conditions, which is suggestive of the formation of intramolecular bonds; there were no apparent multimers formed through intermolecular disulfide bonds, which is consistent with observations of other GST-ADAM disintegrin domain fusion proteins [8, 20, 22, 23, 30, 35]. The purified GST-fertilin βD protein was cleaved with thrombin (Sigma) at 20 μg/ml at 4°C overnight, to separate fertilin β from the GST fusion partner. The cleaved fertilin βD (with the 6-His tag) was separated from GST by chromatography on a glutathione column, and then further purified on a nickel-agarose column (Novagen, Madison, WI). Purified recombinant fertilin βD was dialyzed against WHITCO buffer (109.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 7 mM $NaHCO_3$, 15 mM Hepes) for 36 to 48 h with four or more buffer changes. The dialyzed protein was concentrated (Microcon microconcentrators; Amicon, Beverly, MA) to 2–10 mg/ml. We refer to this protein as "GST-expressed fertilin βD," to indicate that it was expressed as a fusion protein with GST, even though it has been cleaved free of the GST fusion partner for use in this study.

BAP-Presented Peptides

BAP-ECD (also referred to as BAP-βDL [β disintegrin loop]), and its controls BAP-ECE and BAP-ECA, were described previously [15]. The BAP-presented RGD, MLDG, and control peptides were generated using methods previously described [15]. Oligonucleotides were designed corresponding to the sense and antisense strands encoding the desired amino acid sequences (Table 1), and included a *Bgl*II overhang at the 5′ end and a *Sal*I overhang at the 3′ end. The RGD peptide sequence was selected because GRGDTP showed greater activity than GRGDSP in a previous study [41]. All plasmid constructs were sequenced to confirm the insert sequences. The BAP-presented peptides were purified using a nickel agarose column (Novagen) as previously described [15]. The purified BAP-presented peptides were extensively dialyzed against WHITCO buffer, and then concentrated to at least 10 mg/ml.

Egg Collection and Zona Pellucida Removal

This work was conducted in accordance with the *Guiding Principles for Biomedical Research Involving Animals* as promulgated by the Society for the Study of Reproduction. Cumulus cell-free metaphase II-arrested eggs were obtained from superovulated 6- to 9-wk-old CF-1 mice (Harlan, Indianapolis, IN) as previously described [2, 41]. All gamete cultures were performed in Whitten medium [42] containing 22 mM $NaHCO_3$ and 15 mg/ml BSA (Albumax I; Life Technologies, Grand Island, NY) at 37°C in 5% CO_2 in air. Divalent cation concentrations for all incubations were 2.4 mM $CaCl_2$ and 1.2 mM $MgSO_4$. For most experiments, the ZPs were removed by a very brief incubation (≤15 sec) in acidic medium-compatible buffer [2]. For experiments using the monoclonal antibody GoH3 (anti-α_6 integrin subunit; Immunotech, Westbrook, ME), the ZPs were removed by incubating the eggs in medium containing 10 μg/ml chymotrypsin (Sigma) for up to 5 min, allowing the ZP to swell, and then shearing off the ZP with a thin-bore pipette [2, 16]. This treatment is used because GoH3 shows little or no binding to eggs from which the ZPs have been removed by acid solubilization [2]. Following ZP removal, the eggs were allowed to recover for 60 min in Whitten medium.

Luminometric Immunoassay Detection of Protein Binding to ZP-Free Eggs

General information on the luminometric immunoassay to detect the binding of recombinant fertilin proteins diluted in culture medium (referred to as "soluble") have been described elsewhere [2, 15, 39]. In this study, ZP-free eggs were incubated in Whitten medium containing the indicated BAP-presented peptide (20, 40, 80, or 100 μM as noted in figure legends), or the anti-α_6 monoclonal antibody GoH3 (Immunotech; 0.5 mg/ml) or the anti-CD9 monoclonal antibody KMC8.8 (Pharmingen, San Diego, CA; 50–500 μg/ml) for 60 min. The eggs were then incubated with 0.5 mg/ml recombinant fertilin βD (9.0 μM of MBP-fertilin βD, 10.0 μM of MBP-fertilin βDL, or 38.0 μM GST-expressed fertilin βD) in the presence of the indicated BAP-presented peptide or antibody for an additional 60 min. MBP-fertilin βD or -βDL was detected with a rabbit anti-MBP polyclonal serum (New England Biolabs, Beverly, MA) diluted 1:750, followed by 0.12 μg/ml of an alkaline phosphatase (AP)-conjugated goat anti-rabbit immunoglobulin (Ig) G (Jackson Immunoresearch, West Grove, PA). GST-expressed fertilin βD was detected with 100 ng/ml anti His-Tag monoclonal antibody (Novagen), followed by 0.12 μg/ml AP-conjugated goat anti-mouse IgG secondary antibody (Jackson Immunoresearch). AP activity associated with individual eggs was detected by measuring photon emission in raw light units over a 10-sec duration (RLU/10 sec) in a Monolight 3010 luminometer (Analytical Luminescence Laboratory, Sparks, MD) as previously described [2, 15, 39]. Data were normalized for fertilin β binding to eggs treated with the appropriate control (e.g., no or control protein/antibody, noted in the figure legends), defined as 100%. Background levels of luminometric signal (i.e., from eggs with no recom-

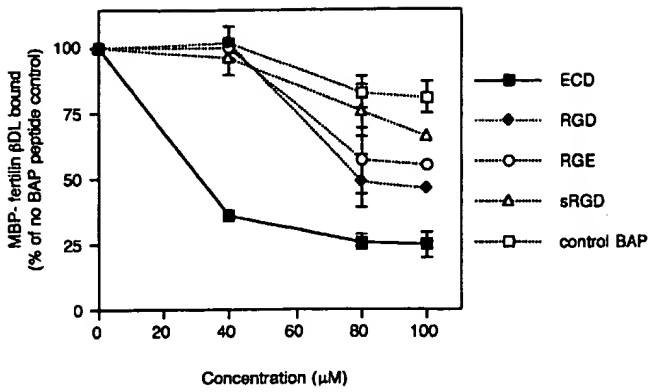

FIG. 2. Effects of BAP-RGD, BAP-RGE, BAP-sRGD, and BAP-ECD on the binding of MBP-fertilin βDL to eggs. ZP-free eggs were incubated in medium containing the indicated concentration (40, 80, or 100 μM) of the indicated BAP-presented peptide for 60 min, and then incubated in medium containing 0.5 mg/ml of MBP-fertilin βDL (10 μM) and the indicated BAP-presented peptide for 60 min. The levels of MBP-fertilin βDL binding to eggs were then assessed with a luminometric immunoassay using an anti-MBP antibody. The data presented are from at least three individual experiments (with 8–12 eggs tested per peptide per experiment), and are normalized for level of MBP-fertilin βDL binding to control eggs with no BAP-presented peptide added (0 μM; average 13 113 ± 1025 RLU/10 sec), which was defined as 100%. (Note that in this assay, background levels of luminometric signal [i.e., from eggs with no recombinant protein but fixed and processed with antibodies then with AP substrate] are ~15%–30% of signals from control eggs incubated in recombinant fertilin β, defined as 100%.) Error bars represent SEM. Filled squares, BAP-ECD; filled diamonds, BAP-RGD; open circles, BAP-RGE; open triangles, BAP-sRGD; open squares, BAP control. The differences between binding levels with 80 or 100 μM BAP-ECD, BAP-RGD, and BAP-RGE were statistically significantly different from those in the absence of BAP-presented peptide and in 80 or 100 μM control BAP. The effects of BAP-sRGD (80 or 100 μM) were not statistically significant compared with these controls.

binant protein but fixed and processed with antibodies and AP substrate) are typically ~15%–30% of signals from control eggs with bound fertilin β [2, 15].

Statistical Analysis

Statistical analyses of data from above-described quantitative luminometric immunoassay were performed by ANOVA with the Fisher protected least significant difference post-hoc testing, performed with StatView version 5.0 (SAS Institute, Cary, NC).

Preparation of Recombinant Fertilin βD-Coated Fluorescent Beads and the Binding of Bead-Immobilized Recombinant Fertilin βD to Eggs

Details on the use of fluorescent beads (0.2 μm yellow-green sulfate-derivatized latex FluoSpheres; Molecular Probes, Eugene, OR) have been described elsewhere [34, 39]. MBP-fertilin βD-coated beads were prepared as previously described for an MBP-fertilin α fusion protein [39]. Beads were coated with GST-expressed fertilin βD by incubating beads in PBS containing 1 mg/ml GST-expressed fertilin βD at 4°C overnight, as described for a GST-expressed version of the cyritestin disintegrin domain [8]. GST-expressed fertilin β-coated beads and MBP-fertilin βD-coated beads bound to eggs to similar extents. GST-expressed fertilin βD-coated beads were then blocked with rabbit IgG (final concentration 1 mg/ml) for 1 h at room temperature. The beads were washed twice with PBS, then resuspended to a concentration of 0.2% in WHITCO buffer. The 0.2% bead suspension was sonicated before use, and then diluted to 0.02% in Whitten medium containing 15 mg/ml BSA. ZP-free eggs were incubated with the indicated BAP-presented peptide (100 μM), or GoH3 or control rat IgG (0.5 mg/ml), or KMC8.8 (50–500 μg/ml) for 60 min. The eggs were then incubated with 0.02% suspension of fertilin β-coated beads in Whitten medium containing the indicated BAP-presented peptide or antibody in a 20-μl culture drop for an additional 60 min. The eggs were washed through four 200-μl drops of Whitten medium containing 15 mg/ml BSA to remove unbound beads, and then were mounted on a microscope slide in Whitten medium and viewed on a Nikon Eclipse fluorescent microscope. Digital images were captured with a Princeton 5 MHz cooled interlined CCD camera (Princeton Instruments Inc., Trenton, NJ) using IP Labs software (Scanalytics, Fairfax, VA). Images for all samples in an experiment were collected with the same exposure time and were not further manipulated, except for cropping in Photoshop 6.0 (Adobe Systems Incorporated, San Jose, CA) for figure preparation. Bead binding levels were assessed qualitatively, as compared to binding levels in control groups, by examining eggs from the entire experimental series (three experiments per series, with 10–20 eggs examined per group per experiment).

RESULTS

Effects of BAP-Presented RGD Peptides on the Binding of MBP-βDL or βD to Eggs

As detailed in the *Introduction,* we hypothesized that fertilin β could interact with a member of the RGD-binding integrin subfamily on the egg membrane. To test this hypothesis, we conducted a series of experiments to examine the effects of BAP-presented RGD and control peptides (RGE and scrambled RGD, sRGD; Table 1) on the binding of soluble MBP-fertilin βDL to eggs. MBP-fertilin βDL (fertilin β disintegrin loop) represents the minimal recognition domain of fertilin β and inhibits sperm-egg binding to a similar extent as recombinant disintegrin domain and the complete extracellular domain of fertilin β [15].

BAP-RGD moderately inhibited the binding of soluble MBP-βDL to eggs, with no inhibition at 40 μM, and inhibition to 46% of control levels at 100 μM (Fig. 2). The positive control BAP-ECD had a much more significant inhibitory effect, with 35% and 25% of control binding levels observed at 40 and 80 μM, respectively. Control BAP and BAP-sRGD had little inhibitory effect on the binding of MBP-βDL to eggs, even at concentrations up to 100 μM. BAP-RGE (the standard negative control for RGD [43]) exhibited a moderate inhibitory effect (inhibition to ~55% of control levels with 100 μM BAP-RGE) on the binding of MBP-fertilin βDL. The effects of 80 and 100 μM BAP-RGD and BAP RGE were not statistically significantly different from each other ($P > 0.05$).

The moderate inhibition of MBP-fertilin βDL binding by BAP-RGE prompted us to design a BAP-RGA peptide (Table 1) in order to assess the effects of an acidic residue in this position, as well as to examine the effects of these various peptides on MBP-fertilin βD binding. At 100 μM, BAP-RGA did not have a significant effect on the binding of MBP-fertilin βD to eggs, similar to BAP-sRGD and control BAP (Fig. 3A). BAP-RGD and BAP-RGE had modest effects, reducing the binding of MBP-fertilin βD to ~57% and ~73%, respectively, of levels observed with control eggs (Fig. 3A). BAP-RGD and BAP-RGE appeared to have somewhat less effect on MBP-fertilin βD binding (Fig. 3A) than on MBP-fertilin βDL binding (Fig. 2). We also compared the effects of BAP-ECE and BAP-ECA to those of BAP-ECD. BAP-ECD reduced MBP-fertilin βD binding to ~40% of control levels, whereas BAP-ECA had no effect (Fig. 3). BAP-ECE had a modest inhibitory effect, reducing the binding of MBP-fertilin βD to ~74% of control levels (Fig. 3A); this effect was statistically different from BAP-ECD and BAP-ECA ($P < 0.05$), and was statistically similar to BAP-RGE ($P > 0.05$).

Effects of BAP-Presented MLDG Peptide on the Binding of MBP-Fertilin βD to Eggs

The partial inhibitory effects observed with BAP-RGE and BAP-ECE (Fig. 3A), with the conservative Glu sub-

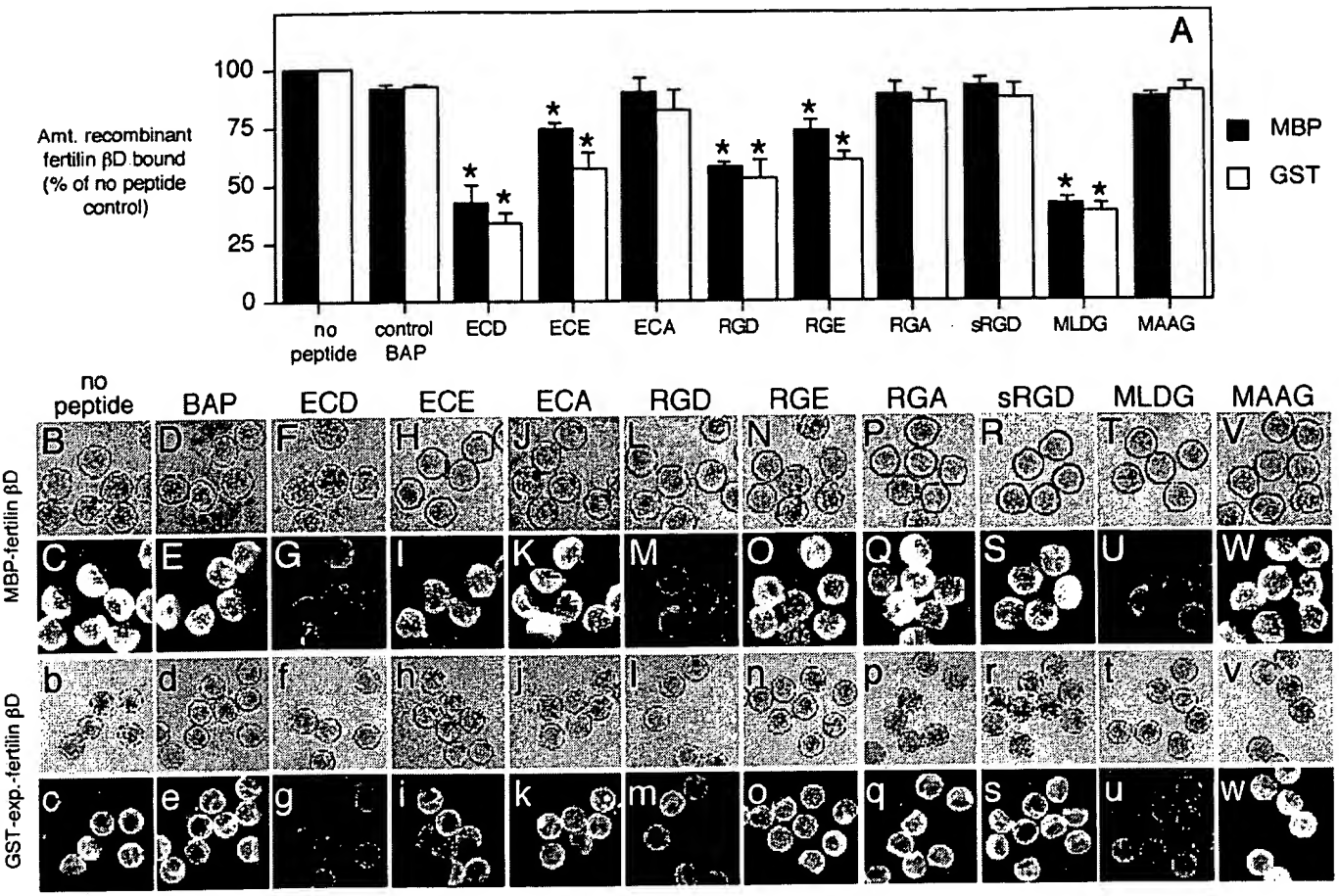

FIG. 3. Effects of BAP-RGD and BAP-MLDG peptides on the binding of MBP-fertilin βD and GST-expressed fertilin βD to eggs. A) ZP-free eggs were incubated in medium containing the indicated BAP-presented peptide (100 μM) for 60 min, and then in medium containing 0.5 mg/ml of MBP-fertilin βD (solid bars) or GST-expressed fertilin βD (open bars) and 100 μM of the indicated BAP-presented peptide for 60 min. Control eggs were incubated in medium lacking BAP-presented peptides ("no peptide"; signals from these eggs were defined as 100%). The levels of MBP-fertilin βD and GST-expressed fertilin βD (with a 6His tag) binding to eggs were then assessed with a luminometric immunoassay using an anti-MBP or anti-6His tag antibody, respectively. The data presented are from at least three individual experiments (with 8–12 eggs tested per peptide per experiment), and are normalized for level of recombinant fertilin βD binding to control eggs ("no peptide" samples; average 21 972 ± 3220 RLU/10 sec and 18 072 ± 3320 RLU/10 sec for the experiments with MBP-fertilin βD and GST-expressed fertilin βD, respectively). Error bars represent SEM. The differences between binding levels with BAP-ECD, BAP-ECE, BAP-RGD, BAP-RGE, and BAP-MLDG were statistically significantly different ($P < 0.05$; denoted by asterisks) from those in the absence of BAP-presented peptide and in control BAP. **B–w**) ZP-free eggs were incubated in 100 μM of the indicated BAP-presented peptide (as labeled in the figure and listed below) for 60 min. The eggs were then cultured for 60 min in a 20-μl drop of Whitten medium containing 100 μM of the indicated BAP-presented peptide and a 0.02% suspension of fluorescent 0.2-μm fluorescent beads coated with MBP-fertilin βD (uppercase letters) or with GST-expressed fertilin βD (lowercase letters). (The diameter of a mouse egg is ~80 μm. Magnification for **B–W** is slightly different from that for **b–w**.) Upper rows of panels (**B–V** and **b–v**) show the phase contrast images; lower rows (**C–W** and **c–w**) of panels show fluorescent images. The egg treatments were as follows: no BAP-presented peptide (**B**, **b**, **C**, and **c**), control BAP (**D**, **d**, **E**, and **e**), BAP-ECD (**F**, **f**, **G**, and **g**), BAP-ECE (**H**, **h**, **I**, and **i**), BAP-ECA (**J**, **j**, **K**, and **k**), BAP-RGD (**L**, **l**, **M**, and **m**), BAP-RGE (**N**, **n**, **O**, and **o**), BAP-RGA (**P**, **p**, **Q**, and **q**), BAP-sRGD (**R**, **r**, **S**, and **s**), BAP-MLDG (**T**, **t**, **U**, and **u**), BAP-MAAG (**V**, **v**, **W**, and **w**). Shown are the results of one experiment that was repeated three times with similar results each time.

stitution for an Asp, on the interaction of fertilin β to eggs were similar to effects seen in a study of VCAM-1, an α_4/α_9 integrin family ligand [44]. In this study, a substitution of GIET (from ICAM-1) for QIDS in VCAM-1 did not adversely affect the ability of VCAM-1 to interact with $\alpha_4\beta_1$ on cells. This relative "tolerance" of Glu for Asp substitutions in $\alpha_4\beta_1$ contrasts members of the RGD-binding family, which interact poorly with ligands with Glu substitutions [45–47]. This prompted us to examine the role of the α_4/α_9 integrin subfamily in the binding of fertilin β to mouse eggs. We generated a BAP-presented peptide containing the sequence MLDG (Table 1) based on the finding that MLDG-containing synthetic peptides and disintegrins EC3 and EC6 inhibit interactions mediated by $\alpha_4\beta_1$ [31] and $\alpha_9\beta_1$ [33]. BAP-MAAG, with alanines substituted for the Leu and Asp residues, was the negative control, based on other studies [33]. As shown in Figures 3A and 4, 100 μM BAP-MLDG inhibited the binding of MBP-fertilin βD to eggs to a very similar extent as did the positive control, BAP-ECD. The concentration dependence of the effect of BAP-MLDG on MBP-fertilin βD binding was virtually identical to that of BAP-ECD (Fig. 4), with a significant ($P < 0.05$) reduction in binding observed with 20–100 μM of BAP-MLDG and BAP-ECD as compared to control BAP; this contrasted the results with BAP-RGD, which showed inhibitory activity only at 80–100 μM (Fig. 2).

Effects of BAP-RGD and BAP-MLDG on the Binding of Bead-Immobilized MBP-Fertilin βD to Eggs

The results in Figures 2, 3A, and 4 show the effects of BAP-RGD and BAP-MLDG on soluble MBP-fertilin βD

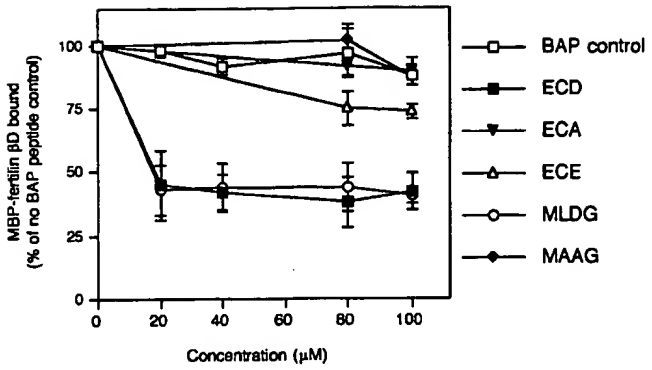

FIG. 4. Concentration dependence of the effects of BAP-ECD and BAP-MLDG on the binding of MBP-fertilin βD to eggs. ZP-free eggs were incubated in medium containing the indicated concentration (20, 40, 80, or 100 μM) of the indicated BAP-presented peptide for 60 min, and then in medium containing 0.5 mg/ml of MBP-fertilin βD (9.0 μM) and the indicated BAP-presented peptide for 60 min. The levels of MBP-fertilin βD binding to eggs were then assessed with a luminometric immunoassay. The data presented are from at least three individual experiments (with 8–12 eggs tested per peptide per experiment), and are normalized for the level of MBP-fertilin βD binding to control eggs with no BAP-presented peptide added (average 21 558 ± 1589 RLU/10 sec), which was defined as 100%. Error bars represent SEM. Open squares, control BAP; filled squares, BAP-ECD; filled triangles, BAP-ECA; open triangles, BAP-ECE; open circles, BAP-MLDG; filled diamonds, BAP-MAAG. The differences between binding levels with 20, 40, 80, or 100 μM BAP-ECD and BAP-MLDG, and 80 or 100 μM BAP-ECE were statistically significantly different ($P < 0.05$) from those in the absence of BAP-presented peptide and in the corresponding concentrations of control BAP. The effects of 80 or 100 μM BAP-MAAG and BAP-ECA were not statistically significant compared with controls.

using a binding assay we have used previously [2, 3, 15, 39, 48]. Other studies have used a different binding assay, with proteins immobilized on fluorescent beads [8, 14, 34], to assess the effects of inhibitory reagents on the interactions of various forms of fertilin β and cyritestin (ADAM3) with mouse eggs. Therefore, we wanted to examine the effects of BAP-RGD and BAP-MLDG on bead-immobilized MBP-fertilin β. In these experiments, the BAP-presented peptides were tested at 100 μM. As shown in Figure 3, BAP-ECD (Fig. 3G) and BAP-MLDG (Fig. 3U) significantly inhibited the interaction of bead-immobilized MBP-fertilin βD to eggs, similar to what was observed with soluble MBP-fertilin βD (Figs. 3A and 4). BAP-ECA (Fig. 3K) had no effect, and BAP-ECE (Fig. 3I) had a partial inhibitory effect, also in agreement with results with soluble MBP-fertilin βD (Figs. 3A and 4, and [15]). It was surprising that results with BAP-RGD and BAP-RGE on bead-immobilized MBP-fertilin βD differed from the results of those with soluble MBP-fertilin βD and βDL (Figs. 2 and 3A). BAP-RGE had little or no effect on the binding of bead-immobilized MBP-fertilin βD (Fig. 3O), with binding levels similar to eggs treated with control BAP, BAP-RGA, and BAP-sRGD (Fig. 3, E, Q, and S). This contrasted the partial inhibitory effect that 100 μM of BAP-RGE had on soluble MBP-fertilin βD and βDL (Figs. 2 and 3A). BAP-RGD (Fig. 3M) inhibited the binding of bead-immobilized MBP-fertilin βD to a very similar extent as BAP-ECD and BAP-MLDG (Fig. 3, G and U). This contrasted the partial inhibitory effect of BAP-RGD on soluble MBP-fertilin βD, which was not nearly as robust as the inhibitory effects of BAP-ECD and BAP-MLDG (Figs. 2–4).

Effects of Anti-α_6 and Anti-CD9 Antibodies on the Binding of Bead-Immobilized MBP-Fertilin βD to Eggs

BAP-RGD and BAP-RGE appeared to have different effects on soluble versus bead-immobilized MBP-fertilin βD. BAP-RGE had no effect on bead-immobilized MBP-fertilin βD (Fig. 3O), but a partial inhibitory effect on soluble MBP-fertilin βD (Figs. 2 and 3A), whereas BAP-RGD had a partial effect on soluble MBP-fertilin βD (Figs. 2 and 3A) but a significant inhibitory effect on bead-immobilized MBP-fertilin βD (Fig. 3M). Likewise, we have observed that another reagent, the anti-CD9 monoclonal antibody KMC8.8, does not affect the binding of soluble MBP-fertilin α (ADAM1) disintegrin domain to eggs, but inhibits bead-immobilized MBP-fertilin αD binding [39]. In light of these data, we wanted to examine the effect of anti-CD9 antibodies on bead-immobilized MBP-fertilin βD. We also examined the anti-α_6 monoclonal antibody GoH3. This antibody has been previously reported to inhibit the binding of some forms of bead-immobilized fertilin β [14, 34] but did not inhibit the binding of soluble full-length recombinant fertilin β [2] or of soluble or bead-immobilized MBP-fertilin αD [39]. Additional data suggest that α_6 is not essential for sperm or fertilin β binding to eggs [2, 18].

We observed that GoH3 (anti-α_6) had very little inhibitory effect on the binding of either soluble or bead-immobilized MBP-fertilin βD to eggs (Fig. 5, A and I), even though GoH3 bound strongly to the egg plasma membrane as detected by immunofluorescence (data not shown). It is interesting that KMC8.8 (anti-CD9) did not have an effect on the binding of soluble MBP-fertilin βD (Fig. 5A), but inhibited the binding of bead-immobilized MBP-fertilin βD (Fig. 5K). KMC8.8 was tested at 50 μg/ml, a concentration that inhibits fertilization [18], and at 500 μg/ml (data not shown). Neither 50 nor 500 μg/ml of KMC8.8 inhibited the binding of soluble MBP-fertilin βD, whereas both concentrations inhibited the binding of bead-immobilized MBP-fertilin βD. The positive control reagent, BAP-ECD, inhibited the binding of soluble (Fig. 5A) and bead-immobilized MBP-fertilin βD (Fig. 5E) when tested in parallel with these antibodies.

Analysis of GST-Expressed Fertilin βD and Effects of Anti-α_6 and Anti-CD9 Antibodies and BAP-RGD and BAP-MLDG on Its Binding to Eggs

The results with GoH3 (Fig. 5I) contrasted with those of a previous study that observed GoH3 inhibition of the binding of recombinant fertilin β prepared as a GST fusion protein [14]. Because we have prepared recombinant fertilin α and β proteins as fusion proteins with MBP [2, 3, 15, 39, 48], we hypothesized that the discrepancies between these results with GoH3 might be due to the method by which recombinant fertilin β was prepared. We therefore generated fusion proteins of GST-fertilin βD, and cleaved fertilin βD from GST with thrombin (Fig. 6), following methods previously described [14, 30].

The results with GST-expressed fertilin βD (Fig. 3, A and b–w) essentially mirrored what was observed with MBP-fertilin βD (Fig. 3, A–W). BAP-RGD moderately inhibited the binding of soluble GST-expressed fertilin βD (Fig. 3A, open bars) and strongly inhibited bead-immobilized GST-expressed fertilin βD (Fig. 3m; on some eggs, this effect was not quite as robust as the effect on MBP-fertilin βD [Fig. 3M]). BAP-RGE had a moderate inhibitory effect on the binding of soluble GST-expressed fertilin βD (Fig. 3A, open bars), but almost no effect on the bind-

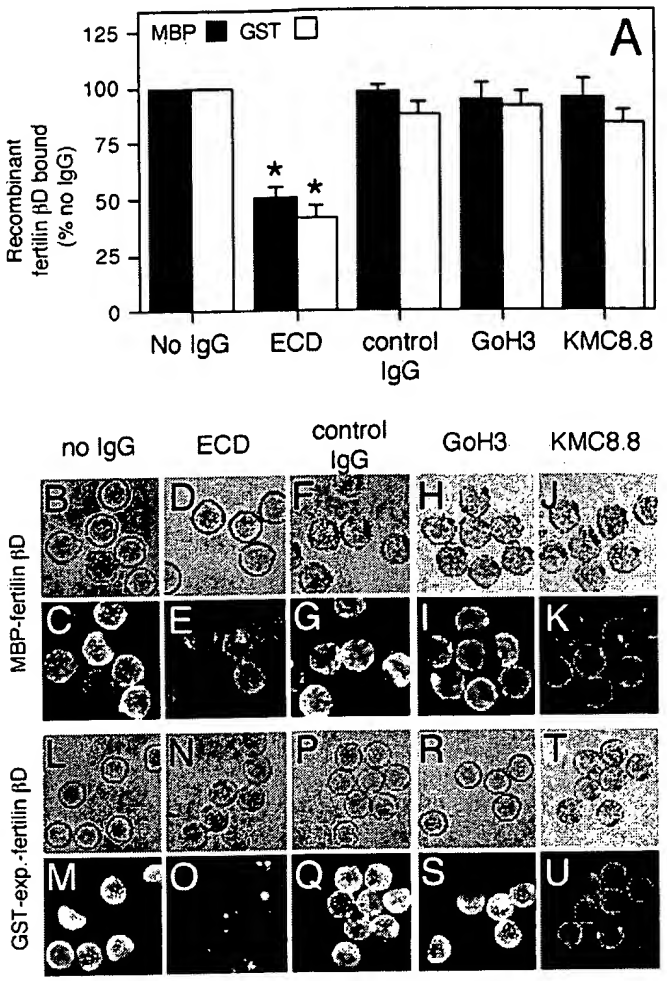

FIG. 5. Effects of anti-α_6 (GoH3) and anti-CD9 (KMC8.8) monoclonal antibodies on the binding of soluble and bead-immobilized MBP-fertilin βD and GST-expressed fertilin βD to eggs. A) ZP-free eggs were incubated in Whitten medium (no IgG) or in medium containing the indicated antibody (500 μg/ml of control nonimmune rat IgG or GoH3, or 50 μg/ml of KMC8.8), or 100 μM BAP-ECD for 60 min. The eggs were then incubated in medium containing 0.5 mg/ml of either MBP-fertilin βD (solid bars) or GST-expressed fertilin βD (open bars) and the indicated antibody or BAP-ECD for 60 min. Egg-associated recombinant fertilin βD was detected using a luminometric immunoassay, using an anti-MBP or anti-6His antibody. The data were normalized for the no IgG control (defined as 100%; average 20604 ± 3169 and 21696 ± 2223 RLU/10 sec for MBP-fertilin βD and GST-expressed fertilin βD, respectively), and represent the results from three separate experiments, with 10–15 eggs examined per experiment per group. The error bars represent the SEM. The differences between no IgG, control IgG, GoH3m, and KMC8.8 were not statistically significant, whereas the difference between no IgG and BAP-ECD were significant (denoted by an asterisk). **B–U)** ZP-free eggs were incubated in Whitten medium (no IgG) or in medium containing the indicated antibody (500 μg/ml of control nonimmune rat IgG or GoH3, or 50 μg/ml of KMC8.8), or containing 100 μM BAP-ECD for 60 min. The eggs were then incubated a 20-μl drop of Whitten medium containing a 0.02% suspension of 0.2-μm fluorescent beads coated with either MBP-fertilin βD (**B–K**) or GST-expressed fertilin β (**L–U**) and the indicated antibody for 60 min. The upper row of panels shows phase contrast images; the lower row of panels shows fluorescent images (**B–K** and **L–U** are slightly different magnifications). The egg treatments were as follows: no IgG (**B, C, L,** and **M**), BAP-ECD (**D, E, N,** and **O**), control nonimmune rat IgG (**F, G, P,** and **Q**), GoH3 (**H, I, R,** and **S**), and KMC8.8 (**J, K, T,** and **U**).

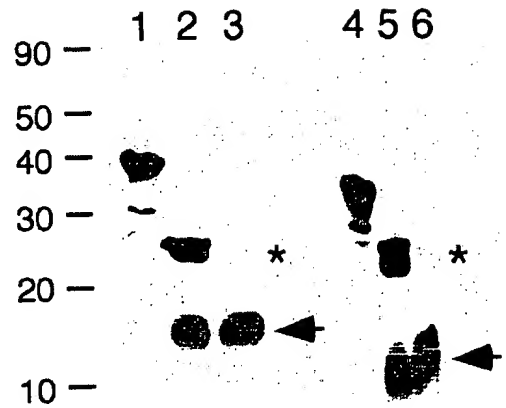

FIG. 6. SDS-PAGE analysis of GST-expressed fertilin βD. The disintegrin domain of mouse fertilin β was generated as GST fusion protein with a 6His tag, which was then released from GST by thrombin cleavage, and purified as described in *Materials and Methods*. Samples were resolved on a 12.5% SDS-polyacrylamide gel under reducing (lanes 1, 2, and 3) or nonreducing (lanes 4, 5, and 6) conditions, and stained with Coomassie Brilliant Blue. Lanes 1 and 4, GST-fertilin βD; lanes 2 and 5, thrombin-cleaved GST-fertilin βD, showing GST (asterisk) and fertilin βD (arrowhead); lanes 3 and 6, purified GST-expressed fertilin βD (arrowhead).

ing of bead-immobilized GST-expressed fertilin βD (Fig. 3o). The effects of BAP-RGD and BAP-RGE on soluble GST-expressed fertilin βD were statistically similar ($P > 0.05$). BAP-MLDG inhibited the binding of both soluble and bead-immobilized GST-expressed fertilin βD (Fig. 3, A and u) to an extent similar to BAP-ECD (Fig. 3, A and g). BAP-ECE had a moderate inhibitory effect on the binding of soluble and bead-immobilized GST-expressed fertilin βD (Fig. 3, A and i). The alanine-substituted and scrambled control peptides (BAP-ECA, BAP-RGA, BAP-sRGD, and BAP-MAAG) had very little effect on the binding of soluble and bead-immobilized GST-expressed fertilin βD to eggs (Fig. 3, A, k, q, s, and w), similar to the control BAP protein (Fig. 3, A and e).

The effects of GoH3 and KMC8.8 on the binding of soluble and bead-immobilized GST-expressed fertilin βD were also similar to the effects of these antibodies on soluble and bead immobilized MBP-fertilin βD (Fig. 5). GoH3 had very little inhibitory effect on the binding of either soluble or bead-immobilized GST-expressed fertilin βD (Fig. 5, A and S). KMC8.8 inhibited the binding of bead-immobilized GST-expressed fertilin βD to eggs (Fig. 5U), but did not appear to affect the binding of soluble GST-expressed fertilin βD at 50 μg/ml (Fig. 5A) or at 500 μg/ml (data not shown). In all experiments, the binding of GoH3 and KMC.8.8 antibodies to eggs was confirmed by indirect immunofluorescence (data not shown). The positive control reagent, BAP-ECD, inhibited the binding of soluble (Fig. 5A) and bead-immobilized GST-expressed βD (Fig. 5O).

DISCUSSION

Candidate Integrins to Serve as Receptors for Fertilin β

Our results with BAP-MLDG suggest the involvement of a member of the α_4/α_9 subfamily of integrins (Fig. 1) in the interaction of fertilin β with the egg membrane. BAP-MLDG, a peptide based on the disintegrin loop sequences of the snake venom disintegrin subunits EC3B and EC6A [31, 33], significantly inhibits fertilin β binding to an extent similar to BAP-ECD, a peptide matching the fertilin β dis-

integrin loop sequence (Fig. 3). EC3, EC6, and MLDG peptides perturb α_4/α_9-mediated interactions, $\alpha_4\beta_1$-VCAM-1, $\alpha_4\beta_7$-MadCAM-1, and $\alpha_9\beta_1$-VCAM-1 [31, 33].

BAP-RGD also inhibits the interaction of fertilin β with the egg membrane, although this inhibition is partial and less robust than inhibition with BAP-MLDG (Figs. 2–4). As noted in the *Introduction,* the RGD-binding integrins and the α_4/α_9 integrins both recognize Asp residues in their ligands (Fig. 1). Interactions between $\alpha_4\beta_1$ and VCAM-1 (mediated by an IDS sequence in VCAM-1) and the CS-1 fragment of fibronectin (mediated an LDV sequence in fibronectin) can be inhibited by specialized forms of RGD or similar Asp-containing peptides [49, 50], although short, linear RGD peptides are ineffective [31, 49]. Thus, there are at least two possible explanations for the activity of BAP-RGD in these fertilin β binding assays. BAP-RGD could be moderately inhibiting an α_4/α_9 integrin on the egg surface to perturb fertilin β binding. It is also possible that an RGD-binding integrin contributes to fertilin β binding; this may be particularly true for bead-immobilized recombinant fertilin β (Fig. 3, M and m; see below). Two α integrin subunits of the RGD-binding family (α_5, α_V) and β subunits with which they dimerize (β_1, β_3, β_5) have been detected on mouse eggs [16, 51–53]. RGD peptides have been reported to inhibit membrane interactions of sperm with hamster or bovine eggs [54, 55] (although they have less effect on mouse gametes [16, 41]), and $\alpha_v\beta_1$ has recently been implicated in the interactions of porcine gametes [56].

The inhibition of interactions by MLDG-containing disintegrins and peptides appears to be quite specific for α_4/α_9 integrins. The heterodimeric disintegrins EC3 (composed of EC3A with the disintegrin loop sequence VGD, and EC3B with MLD) and EC6 (composed of EC6A with MLD, and EC6B with RGD) can inhibit two RGD-binding integrins ($\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$), and an MLDG-containing peptide inhibits $\alpha_5\beta_1$-fibronectin interactions to a modest extent. However, these activities are quite weak, attributable to the VGD or RGD subunits, or both [31, 33]. In contrast, $\alpha_4\beta_1$-VCAM-1 interactions are inhibited by the MLDG-containing EC3B subunit alone [31] or by MLDG-containing peptides [33] but not by RGD-containing peptides [31, 49], and $\alpha_9\beta_1$-VCAM-1 interactions are inhibited by EC3, EC6, and by MLDG-containing peptides, but not by the RGD-containing disintegrin echistatin [33].

Despite the robust effect of BAP-MLDG on the interaction of fertilin β with mouse eggs, the identity of the molecules that are perturbed by BAP-MLDG is unclear. Two obvious possibilities are an α_4 integrin, an α_9 integrin, or both, and reference has been made to the α_9 integrin [30]. The α_4 subunit has been reported to be present on hamster eggs [57], but expression on human eggs is weak or absent [57, 58], and we have failed to detect α_4 on mouse eggs (using two different monoclonal antibodies in immunofluorescence experiments and in highly sensitive luminometric immunoassays; data not shown). It was recently reported that α_9 is detected in a pool of ESTs from three cDNA libraries of unfertilized or fertilized mouse eggs [59], although the protein has not been detected. In addition, it is possible that $\alpha_4\beta_1$ and $\alpha_9\beta_1$ do not recognize an ECD sequence. Although $\alpha_9\beta_1$ apparently binds to ADAM disintegrin domains with DCD, ACD, or SCD sequences [30], an ECD-containing disintegrin (alternagin) has been reported to have no effect on $\alpha_4\beta_1$-VCAM-1 or $\alpha_9\beta_1$-VCAM-1 interactions [60]. Thus, whereas it is possible that fertilin β binds to a known α_4/α_9 integrin, it is also possible that fertilin β binds to a different molecule, potentially a new member of the α_4/α_9 integrin family. These are possibilities we will be pursuing in the future.

We find that GoH3 (anti-α_6 integrin) has very little effect on the binding of recombinant fertilin β to eggs in two different binding assays using two different forms of recombinant fertilin β. We consistently observe strong binding of GoH3 to the egg membrane in these experiments (data not shown). It is unclear what the basis is for the discrepancy between reports of no inhibition by GoH3 (Fig. 5 and [2, 18]) and other reports of GoH3 having inhibitory effects [14, 16, 34]. The concentrations of GoH3 needed to inhibit the binding of sperm and various forms of fertilin β [14, 16, 34] are 10- to 500-fold greater than those needed to inhibit $\alpha_6\beta_1$-laminin interactions [61], and cross-linked complexes of fertilin β disintegrin loop peptide and $\alpha_6\beta_1$ can be immunoprecipitated with GoH3 [17], suggesting that the GoH3 epitope and the peptide binding site do not overlap completely. We speculate that the role of $\alpha_6\beta_1$ in fertilin β binding could be indirect, such as a component of a multimolecular network in the egg membrane (such as a tetraspanin web, as discussed in [8]), or it could represent a novel activity of $\alpha_6\beta_1$ (possibilities for which are discussed in [26]).

Role of CD9, the Effects of Presentation Formats of Fertilin β, and a Model for How Fertilin β Mediates Sperm Adhesion to the Egg Membrane

An anti-CD9 monoclonal antibody (KMC8.8) inhibits fertilin β binding to eggs when recombinant fertilin βD is immobilized on small beads (Fig. 5, K and U) but does not inhibit the binding of soluble recombinant fertilin βD to eggs (Fig. 5A). We have observed similar effects of KMC8.8 on the binding of recombinant fertilin α disintegrin domain to eggs [39]. Moreover, bead-immobilized fertilin βD appears to interact with a site that is sensitive to perturbation by BAP-RGD (Fig. 3, M and m, and Fig. 5L), whereas soluble fertilin β binding is considerably less sensitive to BAP-RGD perturbation (Figs. 2 and 3A). These results indicate that the formats of presentation of recombinant fertilin βD (and fertilin αD as well [39]) affect the sensitivity to inhibition by some reagents. Although the significance of this is not completely clear at this time, we speculate that soluble fertilin βD represents a monovalent ligand and that bead-immobilized fertilin β represents a multivalent ligand (as multiple protein molecules coat a single bead), and that the binding properties of these two types of ligand valencies differ. The interaction of soluble fertilin βD with the egg surface could represent a binding event between individual ligand and receptor molecules, and KMC8.8 may not affect this because the interaction of individual fertilin β molecules (mimicked by this presentation) with one or more binding partners on the egg is not dependent on CD9. The observation that this binding can be perturbed by BAP-MLDG (Fig. 3A) suggest that it could be mediated by an α_4/α_9 integrin or similar molecule (see above). The binding of a fertilin βD-coated bead could be mediated by multiple molecular interactions, which in turn, could lead to adhesion strengthening that allow the bead to remain attached to the egg membrane after a series of washes. This is analogous to cell adhesion systems in which adhesion strengthening can be defined and measured as the ability of an adherent cell (interacting with an substrate via cell adhesion molecules) to resist detaching shear forces. Because the binding of bead-immobilized fertilin βD can

be perturbed by BAP-MLDG and KMC.8.8 (Fig. 3, U and u; and Fig. 5, K and U), and by BAP-RGD to some extent (Fig. 3, M and m), we envision that this could be mediated by an α_4/α_9 integrin and perhaps also an RGD-binding integrin, with these interactions supported by the context of a CD9-containing tetraspanin web in the egg membrane. It is interesting to note that mutations in the α_4 subunit that impair the ability of $\alpha_4\beta_1$ to interact with the tetraspanin CD81 [62] also perturb the strengthening of $\alpha_4\beta_1$-VCAM-1 mediated adhesions, but not the ability of $\alpha_4\beta_1$ to bind to VCAM-1 [63]. Thus, it seems possible that KMC8.8 perturbs the ability of a fertilin β-coated bead to adhere to the egg due to the antibody inducing the dissociation of CD9 from other molecules or by rearranging protein distribution in the CD9-containing tetraspanin-containing network in the lipid bilayer. This could result in a suboptimal organization of sperm ligand receptors and associated molecules in the plane of the egg membrane, leading to reduced binding of bead-immobilized sperm ligands (i.e., fertilin β; Fig. 5, and [35], fertilin α [39], and cyritestin [8]), or of the entire sperm [35]. Thus, these effects of KMC8.8 on multivalent ligands could be indicative of a role in CD9 in the strengthening of adhesions mediated by these and perhaps other sperm ligands. This is an intriguing possibility, particularly in light of the molecular, biochemical, and biophysical parameters that affect complex adhesive events, including the interaction of sperm with egg.

ACKNOWLEDGMENTS

We thank Bayard Storey, William Hanna, and Gregory Kopf for comments on the manuscript.

REFERENCES

1. Myles DG, Kimmel LH, Blobel CP, White JM, Primakoff P. Identification of a binding site in the disintegrin domain of fertilin required for sperm-egg fusion. Proc Natl Acad Sci U S A 1994; 91:4195–4198.
2. Evans JP, Kopf GS, Schultz RM. Characterization of the binding of recombinant mouse sperm fertilin β subunit to mouse eggs: evidence for adhesive activity via an egg β_1 integrin-mediated interaction. Dev Biol 1997; 187:79–93.
3. Evans JP, Schultz RM, Kopf GS. Characterization of the binding of recombinant mouse sperm fertilin α subunit to mouse eggs: evidence for function as a cell adhesion molecule in sperm-egg binding. Dev Biol 1997; 187:94–106.
4. Yuan R, Primakoff P, Myles DG. A role for the disintegrin domain of cyritestin, a sperm surface protein belonging to the ADAM family, in mouse sperm-egg plasma membrane adhesion and fusion. J Cell Biol 1997; 137:105–112.
5. Cho C, Bunch DO, Faure J-E, Goulding EH, Eddy EM, Primakoff P, Myles DG. Fertilization defects in sperm from mice lacking fertilin β. Science 1998; 281:1857–1859.
6. Cho C, Ge H, Branciforte D, Primakoff P, Myles DG. Analysis of mouse fertilin in wild-type and fertilin β −/− sperm: evidence for C-terminal modification, α/β dimerization, and lack of essential role of fertilin α in sperm-egg fusion. Dev Biol 2000; 222:289–295.
7. Nishimura H, Cho C, Branciforte DR, Myles DG, Primakoff P. Analysis of loss of adhesive function in sperm lacking cyritestin or fertilin β. Dev Biol 2001; 233:204–213.
8. Takahashi Y, Bigler D, Ito Y, White JM. Sequence-specific interaction between the disintegrin domain of mouse ADAM3 and murine eggs: role of the β_1 integrin-associated proteins CD9, CD81, and CD98. Mol Biol Cell 2001; 12:809–820.
9. Gould RJ, Polokoff MA, Friedman PA, Huang T-F, Holt JC, Cook JJ, Niewiarowski S. Disintegrins: a family of integrin inhibitory proteins from viper venoms. Proc Soc Exp Biol Med 1990; 195:168–171.
10. McLane MA, Marcinkiewicz C, Vijay-Kumar S, Wierzbicka-Patynowski I, Niewiarowski S. Viper venom disintegrins and related molecules. Proc Soc Exp Biol Med 1998; 219:109–119.
11. Jia LG, Shimokawa K, Bjarnason JB, Fox JW. Snake venom metalloproteinases: structure, function and relationship to the ADAMs family of proteins. Toxicon 1996; 34:1269–1276.
12. Adler M, Lazarus RA, Dennis MS, Wagner G. Solution structure of kistrin, a potent platelet aggregation inhibitor and GP IIb-IIIa antagonist. Science 1991; 253:445–448.
13. Saudek V, Atkinson RA, Pelton JT. Three-dimensional structure of echistatin, the smallest active RGD protein. Biochemistry 1991; 30: 7369–7372.
14. Bigler D, Takahashi Y, Chen MS, Almeida EAC, Osburne L, White JM. Sequence-specific interaction between the disintegrin domain of mouse ADAM2 (fertilin β) and murine eggs: role of the α_6 integrin subunit. J Biol Chem 2000; 275:11576–11584.
15. Zhu X, Bansal NP, Evans JP. Identification of key functional amino acids of the mouse fertilin β (ADAM2) disintegrin loop for cell-cell adhesion during fertilization. J Biol Chem 2000; 275:7677–7683.
16. Almeida EAC, Huovila A-PJ, Sutherland AE, Stephens LE, Calarco PG, Shaw LM, Mercurio AM, Sonnenberg A, Primakoff P, Myles DG, White JM. Mouse egg integrin $\alpha_6\beta_1$ functions as a sperm receptor. Cell 1995; 81:1095–1104.
17. Chen H, Sampson NS. Mediation of sperm-egg fusion: evidence that mouse egg $\alpha_6\beta_1$ integrin is the receptor for sperm fertilinβ. Chem Biol 1999; 6:1–10.
18. Miller BJ, Georges-Labouesse E, Primakoff P, Myles DG. Normal fertilization occurs with eggs lacking the integrin $\alpha_6\beta_1$ and is CD9-dependent. J Cell Biol 2000; 149:1289–1295.
19. Ruoslahti E. RGD and other recognition sequences for integrins. Annu Rev Cell Biol 1996; 12:697–715.
20. Zhang XP, Kamata T, Yokoyama K, Puzon-McLaughlin W, Takada Y. Specific interaction of the recombinant disintegrin-like domain of MDC-15 (metargidin, ADAM-15) with integrin $\alpha_v\beta_3$. J Biol Chem 1998; 273:7345–7350.
21. Nath D, Slocombe PM, Stephens PE, Warn A, Hutchinson GR, Yamada KM, Docherty AJ, Murphy G. Interaction of metargidin (ADAM-15) with $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins on different haemopoietic cells. J Cell Sci 1999; 112:579–587.
22. Cal S, Freije JMP, Lopez JM, Takada Y, Lopez-Otin C. ADAM23/MDC3, a human disintegrin that promotes cell adhesion via interaction with the $\alpha_v\beta_3$ integrin in an RGD-dependent mechanism. Mol Biol Cell 2000; 11:1457–1469.
23. Zhou M, Graham R, Russel G, Croucher PI. MDC-9 (ADAM-9/meltrin γ) functions as an adhesion molecule by binding the $\alpha_v\beta_5$ integrin. Biochem Biophys Res Commun 2001; 280:574–580.
24. Newham P, Craig SE, Seddon GN, Schofield NR, Rees A, Edwards RM, Jones EY, Humphries MJ. Alpha4 integrin binding interfaces on VCAM-1 and MAdCAM-1—integrin binding footprints identify accessory binding sites that play a role in integrin specificity. J Biol Chem 1997; 272:19429–19440.
25. Yokosaki Y, Matsuura N, Higashiyama S, Murakami I, Obara M, Yamakido M, Shigeto N, Chen J, Sheppard D. Identification of the ligand binding site for the integrin $\alpha_9\beta_1$ in the third fibronectin type III repeat of tenascin-C. J Biol Chem 1998; 273:11423–11428.
26. Evans JP. Fertilin β and other ADAMs as integrin ligands: insights into cell adhesion and fertilization. Bioessays 2001; 23:628–639.
27. Wang J-H, Pepinsky RB, Stehle T, Liu J-H, Karpusas M, Browning B, Osborn L. The crystal structure of an N-terminal two-domain fragment of vascular cell adhesion molecule 1 (VCAM-1): a cyclic peptide based on the domain 1 C-D loop can inhibit VCAM-1-α4 integrin interaction. Proc Natl Acad Sci U S A 1995; 92:5714–5718.
28. Tan K, Casasnovas JM, Liu JH, Briskin MJ, Springer TA, Wang JH. The structure of immunoglobulin superfamily domains 1 and 2 of MAdCAM-1 reveals novel features important for integrin recognition. Structure 1998; 6:793–801.
29. Leahy DJ, Aukhil I, Erickson HP. 2.0 A crystal structure of a four-domain segment of human fibronectin encompassing the RGD loop and synergy region. Cell 1996; 84:155–164.
30. Eto K, Puzon-McLaughlin W, Sheppard D, Sehara-Fujisawa A, Zhang X-P, Takada Y. RGD-independent binding of $\alpha_9\beta_1$ to the ADAM-12 and -15 disintegrin domains mediates cell-cell adhesion. J Biol Chem 2000; 275:34922–34930.
31. Marcinkiewicz C, Calvete JJ, Marcinkiewicz MM, Raida M, Vijay-Kumar S, Huang Z, Lobb RR, Niewiarowski S. EC3, a novel heterodimeric disintegrin from *Echis carinatus* venom, inhibits α_4 and α_5 integrins in an RGD-independent manner. J Biol Chem 1999; 274: 12468–12473.
32. Brando C, Marcinkiewicz C, Goldman B, McLane MA, Niewiarowski S. EC3, a heterodimeric disintegrin from *Echis carinatus* ,inhibits human and murine α_4 integrin and attenuates lymphocyte infiltration of

Langerhans islets in pancreas and salivary glands in nonobese diabetic mice. Biochem Biophys Res Commun 2000; 267:413–417.
33. Marcinkiewicz C, Taooka Y, Yokosaki Y, Calvete JJ, Marcinkiewicz MM, Lobb RR, Niewiarowski S, Sheppard D. Inhibitory effects of MLDG-containing heterodimeric disintegrins reveal distinct structural requirements for interaction of the integrin $\alpha_9\beta_1$ with VCAM-1, tenascin-C, and osteopontin. J Biol Chem 2000; 275:31930–31937.
34. Chen MS, Almeida EA, Huovila A, Takahashi Y, Shaw LM, Mercurio AM, White JM. Evidence that distinct states of the integrin $\alpha_6\beta_1$ interact with laminin and an ADAM. J Cell Biol 1999; 144:549–561.
35. Chen MS, Tung KSK, Coonrod SA, Takahashi Y, Bigler D, Chang A, Yamashita Y, Kincade PW, Herr JC, White JM. Role of the integrin associated protein CD9 in binding between sperm ADAM 2 and the egg integrin $\alpha_6\beta_1$: Implication for murine fertilization. Proc Natl Acad Sci U S A 1999; 96:11830–11835.
36. Le Naour F, Rubinstein E, Jasmin C, Prenant M, Boucheix C. Severely reduced female fertility in CD9-deficient mice. Science 2000; 287: 319–321.
37. Miyado K, Yamada G, Yamada S, Hasuwa H, Nakamura Y, Ryu F, Suzuki K, Kosai K, Inoue K, Ogura A, Okabe M, Mekada E. Requirement of CD9 on the egg plasma membrane for fertilization. Science 2000; 287:321–324.
38. Kaji K, Oda S, Shikano T, Ohnuki T, Uematsu Y, Sakagami J, Tada N, Miyazaki S, Kudo A. The gamete fusion process is defective in eggs of Cd9-deficient mice. Nat Genet 2000; 24:279–282.
39. Wong GE, Zhu X, Prater CE, Oh E, Evans JP. Analysis of fertilin α (ADAM1)-mediated sperm-egg cell adhesion during fertilization and identification of an adhesion-mediating sequence in the disintegrin-like domain. J Biol Chem 2001; 276:24937–24945.
40. Rubinstein E, La Naour F, Lagaudriere-Gesbert C, Billard M, Conjeaud H, Boucheix C. CD9, CD63, CD81, and CD82 are components of a surface tetraspan web network connected to HLA-DR and VLA integrins. Eur J Immunol 1996; 26:2657–2665.
41. Evans JP, Schultz RM, Kopf GS. Mouse sperm-egg membrane interactions: analysis of roles of egg integrins and the mouse sperm homologue of PH-30 (fertilin) β. J Cell Sci 1995; 108:3267–3278.
42. Whitten WK. Nutrient requirements for the culture of preimplantation embryos in vitro. Adv Biol Sci 1971; 6:129–139.
43. Pytela R, Pierschbacher MD, Argraves WS, Suzuki S, Ruoslahti E. Arginine-glycine-aspartic acid adhesion receptors. Methods Enzymol 1987; 144:475–489.
44. Osborn L, Vassallo C, Browning BG, Tizard R, Haskard DO, Benjamin CD, Dougas I, Kirchhausen T. Arrangement of domains, and amino acid residues required for binding of vascular cell adhesion molecule-1 to its counter-receptor VLA-4 ($\alpha_4\beta_1$). J Cell Biol 1994; 124:601–608.
45. Cherny RC, Honan MA, Thiagarajan P. Site-directed mutagenesis of the arginine-glycine-aspartic acid in vitronectin abolishes cell adhesion. J Biol Chem 1993; 268:9725–9729.
46. Obara M, Kang MS, Yamada KM. Site-directed mutagenesis of the cell-binding domain of human fibronectin: separable, synergistic sites mediate adhesive function. Cell 1988; 53:649–657.
47. Dennis MS, Carter P, Lazarus RA. Binding interactions of kistrin with platelet glycoprotein IIb-IIIa: analysis by site-directed mutagenesis. Proteins 1993; 15:312–321.
48. Evans JP, Schultz RM, Kopf GS. Roles of the disintegrin domains of mouse fertilins α and β in fertilization. Biol Reprod 1998; 59:145–152.
49. Cardarelli PM, Cobb RR, Nowlin DM, Scholz W, Gorcsan F, Moscinski M, Yasuhara M, Chiang SL, Lobl TJ. Cyclic RGD peptide inhibits $\alpha_4\beta_1$ interaction with connecting segment 1 and vascular cell adhesion molecule. J Biol Chem 1994; 269:18668–18673.
50. Nowlin DM, Gorcsan F, Moscinski M, Chiang S-L, Lobl TJ, Cardarelli PM. A novel cyclic pentapeptide inhibits $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin-mediated cell adhesion. J Biol Chem 1993; 268:20352–20359.
51. Sutherland AE, Calarco PG, Damsky CH. Developmental regulation of integrin expression at the time of implantation in the mouse embryo. Development 1993; 119:1175–1186.
52. Tarone G, Russo MA, Hirsch E, Odorisio T, Altruda F, Silengo L, Siracusa G. Expression of β1 integrin complexes on the surfaces of unfertilized mouse oocyte. Development 1993; 117:1369–1375.
53. Evans JP, Schultz RM, Kopf GS. Identification and localization of integrin subunits in oocytes and eggs of the mouse. Mol Reprod Dev 1995; 40:211–220.
54. Bronson RA, Fusi F. Evidence that an Arg-Gly-Asp adhesion sequence plays a role in mammalian fertilization. Biol Reprod 1990; 43:1019–1025.
55. Campbell KD, Reed WA, White KL. Ability of integrins to mediate fertilization, intracellular calcium release, and parthenogenetic development in bovine oocytes. Biol Reprod 2000; 62:1702–1709.
56. Linfor J, Berger T. Potential role of α_v and β_1 integrins as oocyte adhesion molecules during fertilization in pigs. J Reprod Fertil 2000; 120:65–72.
57. de Nadai C, Fenichel P, Donzeau M, Epel D, Ciapa B. Characterisation and role of integrins during gametic interaction and egg activation. Zygote 1996; 4:31–40.
58. Campbell S, Swann HR, Seif MW, Kimber SJ, Aplin JD. Cell adhesion molecules on the oocyte and preimplantation human embryo. Hum Reprod 1995; 10:1571–1778.
59. Stanton JL, Green DLP. A set of 840 mouse oocyte genes with well-matched human homologues. Mol Human Reprod 2001; 7:521–543.
60. Souza DH, Iemma MR, Ferreira LL, Faria JP, Oliva ML, Zingali RB, Niewiarowski S, Selistre-de-Araujo HS. The disintegrin-like domain of the snake venom metalloprotease alternagin inhibits $\alpha_2\beta_1$ integrin-mediated cell adhesion. Arch Biochem Biophys 2000; 384:341–350.
61. Sonnenberg A, Modderman PW, Hogervorst F. Laminin receptor on platelets is the integrin VLA-6. Nature 1988; 336:487–489.
62. Mannion BA, Berditchevski F, Kraeft SK, Chen LB, Hemler ME. Transmembrane-4 superfamily proteins CD81 (TAPA-1), CD82, CD63, and CD53 specifically associated with integrin $\alpha_4\beta_1$ (CD49d/CD29). J Immunol 1996; 157:2039–2047.
63. Pujades C, Alon R, Yauch RL, Masumoto A, Burkly LC, Chen C, Springer TA, Lobb RR, Hemler ME. Defining extracellular integrin α-chain sites that affect cell adhesion and adhesion strengthening without altering soluble ligand binding. Mol Biol Cell 1997; 8:2647–2657.
64. Camper L, Hellman U, Lundgren-Akerlund E. Isolation, cloning, and sequence analysis of the integrin subunit α_{10}, a β_1-associated collagen binding integrin expressed on chondrocytes. J Biol Chem 1998; 273: 20383–20389.
65. Velling T, Kusche-Gullberg M, Sejersen T, Gullberg D. cDNA cloning and chromosomal localization of human α_{11} integrin. A collagen-binding, I domain-containing, β1-associated integrin α-chain present in muscle tissues. J Biol Chem 1999; 274:25735–25742.